QUANTITATIVE DETERMINATION OF BENZO[A]PYRENE IN TAR-CONTAINING DRUGS BY AN ISOTOPE DILUTION METHOD

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16. Abstract Studies were made of a method of quantitative determination of the benzo[a]pyrene contained in tar-containing drugs by an isotope dilution method using tritiumized benzo[a]pyrene. Analytical procedures were performed in the following sequence: liquid phase distribution extraction method, dual-layer, one-dimensional thin layer chromatography and ultraviolet absorption spectral analysis. The rate of recovery obtained at this time varied between 30% and 80%, with a fluctuation coefficient of 15-30%. If this were corrected by the recovery ratio, the amount of benzo[a]pyrene in the sample was found in a state close to 100%, and the fluctuation coefficient was reduced to 4-9%. Even in cases when benzo[a]pyrene is not detected in a sample, its ascertainable limit can be found easily. The benzo[a]pyrene contents in pine tar, Pityrol pasta, Glyteer, and Ichthammol were 9.72, 1.32, 12.90 ppm and less than 0.05 ppm, respectively.

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QUANTITATIVE DETERMINATION OF BENZO[A]PYRENE IN TAR-CONTAINING DRUGS BY AN ISOTOPE DILUTION METHOD

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1. Introduction

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Many determinations have been made thus far of benzo[a]pyrene (BP), a carcinogenic substance contained in minute quantities in samples. Among them, there are a number of reports in which the BP contents have been sought by means of the isotope dilution method using BP labeled with ^{14}C [1-3]. However, since it is impossible to obtain a high specific activity with ^{14}C markers, they are unsuitable for the determination of BP, which is present only in minute quantities. When the writers analyzed BP by the isotope dilution method, using tritiumized BP (^{3}H-BP) with a high specific activity, which is sold on the market, they found that the amount of ^{3}H-BP to be added could be extremely small. Consequently, it was possible to make the determinations without considering the ^{3}H-BP which had been added, and accurate values were also obtained. These findings are reported herein. Since the report of Hirohata et al. [4] is the only case where the aromatic polycyclic hydrocarbons in tar-containing drugs have been analyzed, this method was applied to seek the amounts of BP in these drugs.

2. Experiments

2.1. Reagents

Solvent: Special grade solvent was distilled and refined.

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Hydrochloric acid: Special grade.

Anhydrous sodium sulfate: Special grade.

Alumina: 200-300 mesh (manufactured by Wako Seiyaku).

Florisil: 60-100 mesh (manufactured by Katayama Kagaku).

Silica gel: Silica gel G for thin-layer chromatography (manufactured by E. Merck).

Acetyl cellulose: Acetyl cellulose for thin-layer chromatography. Acetylation ratio 20 + 30% (1:1) (manufactured by Macherey Nagel).

BP: Standard BP was that manufactured by L. Light which had been recrystallized in benzene-methanol.

^{3}H-BP: Benzene solution of BP generally leveled with tritium with a specific activity of 5.8 Ci/mM and a radiochemical purity of 96% or higher was purchased from the Japan Radioisotope Society. Before use, it was purified by column chromatography using alumina with 4% water content (30 g) and cyclohexane. When the specific activity of the ^{3}H-BP was sought before and after column chromatography by measuring the mass by means of the ultraviolet absorption spectrum and by measuring the radioactivity by a liquid scintillation counter, no changes in its intensity were found.

Scintillator: Prepared by dissolving 4 g of PPO (manufactured by Hakuto) and 0.4 g of dimethyl POPOP (manufactured by Beckman) in 1 ℓ of toluene.

2.2. Devices and Equipment

Devices for thin-layer chromatograph: The applicator used (manufactured by Mitamura Riken) was improved for two-layer use. Two-layer chromatoplates of silica gel G (4 x 20 cm) and acetyl cellulose (16 x 20 cm) were prepared on the same glass plate (20 x 20 cm) following the method of Matsushita et al. [5]. The samples were spotted using a 50 µl microsyringe (manufactured by Jintan Thermo) and were developed with a vertical developing layer (manufactured by Toyo Kagaku Sangyo).

Ultraviolet irradiating lamp: Manasuru light 365 nm.

Recording spectrophotometer: Shimadzu MPS-50L.

Liquid scintillation counter: Beckman LS-100 using glass sample bottles (Hakuto extra vials). The counting efficiency was sought by the outside ray source method.

2.3. Samples

We used tar-containing drugs which are being sold on the market. They were pine tar, Pityrol pasta, Glyteer and Ichthammol.

2.4. Analytical Procedures

Exactly 10 g of the sample was measured out, a definite amount of a cyclohexane solution of ^{3}H-BP (approximately 50,000 dpm) was added, and they were mixed well. Since there are some samples which do not dissolve easily, 75 ml of methanol-water (4:1) was added to these, and they were then stirred well. The entire quantity was transferred into a separating funnel, and extraction was performed three times with 50 ml of cyclohexane.

The cyclohexane extract was further extracted with dimethyl sulfoxide (50 ml x 5). The dimethyl sulfoxide solution was diluted with the same amount of 15% hydrochloric acid, and extraction was performed again with cyclohexane (100 ml x 3). After this, cyclohexane solution was dried by means of anhydrous sodium sulfate, it was passed through a column of 50 g of Florisil. The flashed cyclohexane was thrown away. Then 500 ml of the eluted benzene was collected and concentrated. It was then coated in lines on the silica gel layers of two thin-layer chromatoplates. These plates were developed by means of a mixed solvent of ethanol-toluene-water (17:4:1). The band corresponding to BP [6] in the vicinity of R_f 0.4, which displays an intense violet fluorescence when exposed to an ultraviolet irradiating lamp, was scraped off, collected on a cotton stopper funnel, and extracted by means of benzene (20 ml). The benzene extract was passed through a column with a small amount of Florisil (1 g) to remove the acetyl cellulose. This column was further washed out with 20 ml of benzene, and the distillates were combined and concentrated. Then they were dried in a silica gel desiccator. A definite amount (10 ml) of cyclohexane was added to this to dissolve it, and part of it (1 ml) was measured in the liquid scintillation counter, while the ultraviolet absorption spectrum was measured with the remainder (Fig. 1). Measurement of the mass of the BP was made by the base line method [7, 8], using the absorption maximum of 384.5 nm.

3. Results and Discussion

3.1. Study of the Effects of the Sample and Solvent on the Counting Efficiency

An examination of the ultraviolet absorption spectrum for the portion of the BP recovered in the section on the experiments (Fig. 1) reveals that unseparated impurities are also contained in addition to BP. Furthermore, cyclohexane is put into the

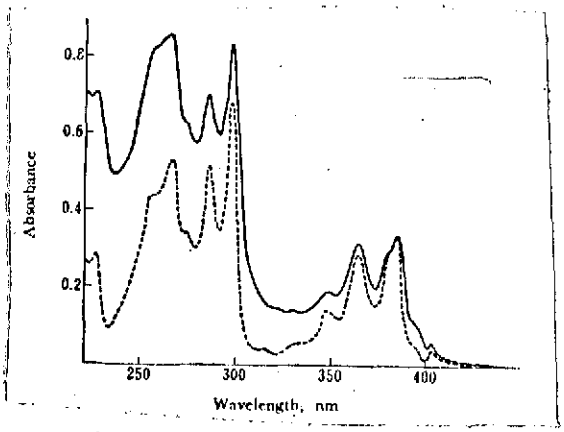

Fig. 1. Ultraviolet absorption spectra of benzo[a]-pyrene (dotted line) and benzo[a]pyrene fraction from pine tar (solid line).

sample bottles during measurement of the count values. A study was made of the effects on the counting efficiency of these when they are mixed in the scintillator. A definite amount of ^{3}H-BP was taken in the sample bottles together with the scintillator and was measured in the scintillation counter. Small amounts of the cyclohexane solution of the portion of BP separated similarly from the samples without adding ^{3}H-BP were added to these sample bottles, and the count values were measured. Small amounts of cyclohexane were also added to separate sample bottles, and the count values were measured. In all cases, the counting efficiency declined following the standard quenching curves of this counter. In other words, the sample and solvent used here do not have any abnormal effect on the counting efficiency.

3.2. Ascertainable Limits of BP in Samples

In cases when a 1-cm cell is used with the ultraviolet absorption spectrum method, at a BP concentration of 0.05 μg/ml, there will be an absorption of 384.5 nm, or an absorbance of 0.005, and the presence of BP will be observed. The quantity of ^{3}H-BP of 50,000 dpm, calculated by its specific activity or by absorption photometry, will be approximately 1 ng. That is, since the amounts of ^{3}H-BP added in each of the experiments are extremely small, they will exert no effect at all on the recovered ultraviolet absorption spectra. It is clear from the facts mentioned above that when the BP in a certain sample is determined by this isotope dilution method and no absorption at all can be

seen at 384.5 nm in the BP portion, the BP in the given sample will have a concentration below the ascertainble limits of BP as calculated by the following equation.

$$X = M \times \frac{V}{W} \times \frac{100}{R}$$

X: Ascertainable limits of BP in sample (μg/g);
M: Minimum BP concentration which can be ascertained by means of absorption spectrum (μg/ml)
V: Amount of solvent with dissolved BP portion (ml)
W: Amount of sample used (g)
R: ^{3}H-BP recovery ratio (%)

3.3. Recovery Tests

The following problems are at issue when this isotope dilution method is applied to the determination of BP in tar-containing drugs. (1) The ^{3}H-BP which is added is subjected in advance to column chromatography to remove the decomposition products which may possibly be present. However, even in this method impurities which have not yet been separated may still be present. (2) When the ^{3}H-BP is mixed with the sample, both are dissolved in the solvent and are stirred well. However, there /1426 still remain small amounts in the sample which are not dissolved in the solvent. Therefore, it is possible that the marker may be present unevenly in the sample. (3) The finally recovered BP is refined by means of thin-layer chromatography. However, as is shown in Fig. 1, it does not give exactly the same absorption spectrum as the standard BP. That is, it still contains impurities even at the final stage. It was desired to discover whether these problems have any effect on the actual determination or not, and if so, to what extent? In order to investigate these matters, a definite amount of BP was added to the sample, the aforementioned analytical method was repeated, and recovery tests were performed. Amounts of 33.0 μg and 4.4 μg of BP were added,

using Ichthammol as the sample, it having been ascertained by the method in Section 3.2 that its BP contents are less than 0.05 ppm. The results are as shown in Table I. In all cases, BP quantities close to the amounts added were detected. That is, it is possible to find out accurately the amount of BP in a sample by this method without any great influence by the problem mentioned above.

TABLE I. RECOVERY OF BENZO[A]PYRENE ADDED TO ICHTHAMMOL

Amount added (μg)		Found 1	2	3	4	5	Average	Standard deviation	Deviation coefficient (%)
33.0	a	38.6	28.6	34.8	35.7	33.9	34.3	3.7	10.6
	b	(53.6)	(83.9)	(56.3)	(69.5)	(90.0)	(70.7)	(16.2)	(22.9)
4.4	a	4.6	4.5	4.8	4.9	4.5	4.7	0.2	4.0
	b	(75.8)	(84.3)	(74.5)	(82.1)	(84.3)	(80.2)	(4.7)	(5.9)

a: Amount (μg) recovered and corrected by the isotope dilution method; b: Recovery ratio (%) obtained by counting the isotope

3.4. BP Contents in Tar-Containing Drugs

The BP contents in four different tar-containing drugs: pine tar, Pityrol pasta, Glyteer, and Ichthammol, were analyzed five times each by this isotope dilution method. The results are as shown in Table II. The mean values were 9.72, 1.32, 12.90 ppm, and less than 0.05 ppm, respectively. The recovery ratios of each experiment were found from the ^{3}H-BP added (Table II). According to these results, the ratio was within the range of 30-80%, so that there are cases when the recovery ratio is quite poor. The coefficients of variation in these cases were 15-30%. In other words, the values sought from the ultraviolet absorption spectra would have dispersions of about this degree. However, if these values were corrected by the respective recovery ratios, the true BP contents of close to 100% would be obtained, and the coefficients of variation would decline to 4-9%.

TABLE II. CONCENTRATION AND RECOVERY OF BENZO[A]PYRENE IN TAR-CONTAINING DRUGS

Tar contg. drug		Found 1	2	3	4	5	Average	Standard deviation	Deviation coefficient (%)
Pine tar	a	9.26	9.64	10.22	9.38	10.11	9.72	0.43	4.4
	b	(64.8)	(47.3)	(62.6)	(69.7)	(56.2)	(60.1)	(8.7)	(14.4)
Fityrol pasta	a	1.35	1.22	1.39	1.45	1.19	1.32	0.11	8.5
	b	(78.0)	(77.1)	(82.8)	(79.5)	(51.5)	(73.8)	(12.6)	(17.0)
Glyteer	a	13.8	12.0	13.8	12.0	12.9	12.9	0.90	6.9
	b	(59.4)	(30.0)	(48.4)	(66.4)	(39.8)	(48.6)	(14.4)	(29.6)
Ichthammol	a	<0.09	<0.08	<0.06	<0.07	<0.05			
	b	(28.0)	(30.0)	(42.5)	(35.1)	(55.5)	(38.2)	(11.2)	(29.3)

a : Concentration (ppm) of benzo[a]pyrene in the drug; b : Recovery ratio (%) obtained by counting the isotope

4. Conclusion

The writers have thus far been engaged in analysis of the BP and other aromatic polycyclic hydrocarbons contained in various samples [6, 9, 13]. According to their findings, quite lengthy analytical procedures are necessary in order to analyze BP. For this reason, there are cases when the BP recovery ratio is poor. The BP recovery ratio in each of the analytical procedures has been found by performing recovery experiment separately. However, it is not known whether analysis is always being performed at the given recovery ratio. Even in these experiments, there is a fluctuation of 30-80% in the recovery ratios of the ^{3}H-BP. If data containing errors such as these are used to make comparisons of the state of pollution in various areas or to compare the carcinogenic properties originating in BP, it is possible that erroneous interpretations may be formulated. However, a more accurate causal relationship can be discovered by analyzing by the isotope dilution method, since the BP contents in the samples can be found in a state close to 100%.

The isotope dilution method with ^{14}C-BP which was used thus far has a low specific activity. For this reason, larger amounts would be added, and the absorption spectra based on ^{14}C-BP would appear. Thus, this method is unsuitable for the analysis of

minute amounts of BP. However, it is possible to obtain extremely high specific activities of ^{3}H-BP, and only very small amounts need to be added in order to obtain the needed count values. /1427
Consequently, the added ^{3}H-BP will have no influence whatever on the absorption spectrum. In the isotope dilution method using ^{3}H-BP, if it is possible to measure the absorption spectrum of the minute amounts of BP in the sample, it will be possible to arrive at an accurate quantitative determination of it. Even when BP is not detected in the absorption spectrum, it is possible to seek the ascertainable limits indicating the point beyond which BP will not be present in the sample. This can be done without ascertaining this by repeated experimentation.

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